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PATENT

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Applicant: Richard Voellmy

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Filed: May 3, 1999 Examiner: Dr. Ulrike Winkler

For: MOLECULAR REGULATORY CIRCUITS TO ACHIEVE SUSTAINED ACTIVATION OF INTEREST BY A SINGLE STRESS

CERTIFICATE OF MAILING

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On 4/12/01 R. Voellmy
Date Signature

Richard Voellmy

DECLARATION OF RICHARD VOELLMY, Ph.D., UNDER 37 C.F.R. 1.132

Assistant Commissioner for Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Dr. Richard Voellmy of 701 Brickell Key Blvd., Miami, FL 33131 (mailing address: Dept. of Biochemistry & Molecular Biology, University of Miami School of Medicine, 1011 N.W. 15th Street, Miami, FL 33136), declare and state that:

1. I am a Professor of Biochemistry & Molecular Biology at the University of Miami School of Medicine.

2. I received a Bachelor of Science degree (Diploma) in Microbiology and Biochemistry in 1971 and a Ph.D. in Microbiology in 1975 from the Swiss Federal Institute of Technology in Zuerich, Switzerland (ETH). From 1975-1978 I was a Research Associate and a Senior Research Associate in the Department of Physiology of Harvard Medical School and from 1978-1982 I was a Postdoctoral Fellow in the Department of Molecular Biology of the University of Geneva, Switzerland. In 1982 I was appointed an Assistant Professor, in 1983 an Associate Professor and in 1987 a Full Professor of Biochemistry & Molecular Biology at the University of Miami. Over the last twenty years I authored numerous peer-reviewed articles relating to the regulation of the stress response.

3. I am the inventor of the referenced application and am thoroughly familiar with the subject matter described in the referenced application. I am familiar with the prosecution history as well as with the rejections in the most recent Office Action dated December 19, 2000.

4. This declaration addresses new rejections made by the Examiner under 35 U.S.C. 112, first paragraph. In this declaration I report experiments performed under my direction showing that regulatory circuits can be prepared using only information provided in the referenced application in combination with readily available public information and routine subcloning technology that possess the properties of the circuits of the invention. Four different transcription factors were used to prepare five operational regulatory circuits of the invention. The five circuits include examples of type 1, type 2 as well as type 3 circuits of the invention.

5. The basic circuits described in the referenced application (also referred to below as "the present invention" or "the invention") are most readily characterized as consisting of a transcription factor whose expression is under the control of a stress-activatable promoter and of a promoter that responds to the transcription factor itself, and a gene of interest under the control of a promoter that is responsive to the transcription factor. Expression of the transcription factor can be from a single gene linked to a promoter that is both

stress- and transcription factor-activatable or from two genes, one of which is stress-activatable and the other activatable by the transcription factor (p.3 and p.4, lines 15-23, of the referenced application). The circuits of the present invention provide for the sustained activation of a gene of interest by a single, transient stress such as a brief heat treatment.

A transcription factor useful in the invention is a protein that binds to a nucleic acid and thereby influences its transcription by altering rates of transcription initiation or elongation. See page 8, lines 28-30. Hence, a transcription factor useful in the invention is defined functionally. This implies that binding of the transcription factor to a nucleic acid as well as its transcriptional activity were previously assessed experimentally. Hence, nucleic acid binding sites and other sequences required for transactivation by the transcription factor are already known at the time of use of the factor in a circuit of the invention.

MATERIALS AND METHODS

Plasmids and constructions

Construct CMV-HSF1(+) was prepared by subcloning a HindIII-EcoRI fragment from a pGem3Z(+)-derived plasmid containing sequences coding for HSF1d202-316 (Zuo et al., 1994) in pcDNA3.1 (Invitrogen). To construct hsp70-HSF1(+), the above HindIII-EcoRI fragment was inserted in between the unique HindIII and EcoRI sites of construct CMV-hsp70B. CMV-hsp70B was prepared by digestion of pcDNA3.1 with BglII and HindIII, and ligation of the larger fragment to a BglII-HindIII fragment from pOR173 containing promoter and RNA leader sequences from the human *hsp70B* gene (Voellmy et al., 1985). Reporter gene construct hsp70-Luc was obtained by subcloning promoter and RNA leader sequences of the human *hsp70B* gene from pOR173 into a plasmid containing a firefly luciferase gene, and lexA-Luc by transferring a fragment containing promoter and RNA leader sequences from LexA-CAT (Zuo et al., 1994; 1995) to pGL2B (Promega). pGem-LexA was

prepared by digestion of LexA-CAT DNA with PstI/SmaI, addition of a synthetic HindIII linker, and digestion with HindIII. An about 500bp-long fragment including LexA-binding elements was ligated to PstI/HindIII-digested pGem3Z(+) DNA. To construct lexA-LexA-HSF1(+), pcDNA3.1 was digested with BglII and, after filling in ends with Klenow DNA polymerase, with HindIII, and the large vector fragment was ligated to the smaller of the fragments generated by a SmaI/HindIII digest of pGem-LexA DNA. The resulting plasmid was digested with HindIII and EcoRI, and the larger fragment was ligated to a HindIII-EcoRI fragment containing sequences coding for LexA-HSF1d202-316 isolated from a pGem3Z(+)-derived plasmid containing the sequences (Zuo et al., 1994). To construct hsp70-LexA-HSF1(+), a HindIII-EcoRI fragment from a pGem3Z(+)-derived plasmid containing sequences coding for LexA-HSF1d202-316 (Zuo et al., 1994) was inserted in between the unique HindIII and EcoRI sites of construct CMV-hsp70B. To prepare pcDNA3.1-GLVP, a KpnI-BamHI fragment from plasmid pCEP4-GLVP (Wang et al., 1994, 1997a & b) containing sequences coding for GLVP was subcloned in pcDNA3.1. GLVP sequences and adjacent BGH polyA sequences were PCR-amplified using primers 5'-GCCTTTAAACTTAAGCTTGGT-3' and 5'-GAAGCCCCTAGGCCCACC GC-3'. The blunt-ended PCR product was digested with AvrII and ligated to a HindIII-XhoI(filled) fragment from p17x4 TATA CAT (Wang et al., 1994, 1997a & b) including a GAL4-responsive promoter, and the ligation product was inserted in between the HindIII and XbaI sites of SP72 (Promega). The resulting construct was designated 17x4-GLVP. To prepare hsp70-GLVP, the above PCR product was digested with AvrII and inserted in between the HindIII(filled) and XbaI sites of SP72-hsp70B. SP72-hsp70B had been constructed by insertion of an XhoI-HindIII fragment containing *hsp70B* gene promoter sequences from pOR173 (Voellmy et al., 1985) in between the XhoI and HindIII sites of pSP72 (Promega). To construct p17x4-Luc, a HindIII(filled)-XhoI fragment from p17x4 TATA CAT (Wang et al., 1994, 1997a & b) including a GAL4-responsive promoter was inserted in between

the SmaI and XhoI sites of pGL2B. To prepare pCR-Blunt-VgEcR/RXR, a fragment (about 5.5 kbp in length) containing VgEcR-coding sequences as well as an independent RXR gene was PCR-amplified from pVgRXR (Invitrogen) using primers 5'ATTTATCCTAGGCAGAA TTCAGGGTTTAA AGCC and 5'ATTTATCCTAGGTGGTTCTTTCCGCCTCAG and inserted into vector pCR-Blunt (Invitrogen). Plasmid hsp70-VgEcR/RXR was constructed by insertion of an AvrII fragment from pCR-Blunt-VgEcR/RXR containing VgEcR and RXR sequences into the XbaI site of pSP72-hsp70B. Plasmid E/GRE-VgEcR was obtained by PCR amplification from pVgEcR/RXR of a fragment containing VgEcR-coding sequences using primers 5'TGGATATCTGCAGAATTCAGGGTTTAAAGCC and 5'TATTTATGCGGCCGCTCCAAGCTGGGTACGATCGAG, digestion with EcoRI and NotI and ligation with EcoRI/NotI-digested pIND (Invitrogen) DNA. Plasmid E/GRE-Luc was constructed by ligation of a HindIII-SalI fragment from pGL3B (Promega) including luciferase-coding sequences and HindIII/XhoI-digested pIND DNA.

Cell culture and transfections

For human HeLa cell line HeLa-CAT and its cultivation see Baler et al. (1992). Cell culture reagents were from Life Technologies. Actinomycin D, cycloheximide and mifepristone were from Sigma, and ponasterone A from Invitrogen. For heat treatment, culture dishes were immersed in a temperature-controlled water bath. Transfections of 90%-confluent cell cultures were carried out using Lipofectamine^{TM} reagent (Life Technologies). Subsequent to transfection, medium was replaced, and cells incubated under standard conditions and with daily changes of medium for the indicated time periods. The reproducibility of the transfection procedure was ascertained in preliminary experiments.

Measurement of luciferase activity

Cells were lysed and assayed for luciferase activity using Luciferase Assay System Freezer-1 Pack kit (Promega) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) according to the manufacturers' protocols. Protein concentrations of lysates were determined using the Bio-Rad Protein Assay kit (Biorad Laboratories) to permit normalization of luciferase activities. All experiments were repeated at least twice, and each data point was derived from assays of 3 parallel samples. Mean values and standard deviations were calculated. Luciferase activities shown in the figures are expressed relative to control data points ("fold induction").

Levels of human growth hormone were measured by ELISA.

RESULTS

Type 1 Circuit

The first regulatory circuit described under this heading is of the exact same type as that discussed in the referenced application on pp.12-15, under Example 1 and in Fig.1.

Heat activation of *hsp* genes in vertebrate cells is mediated by heat shock transcription factor 1 (HSF1) (Voellmy, 1996). This factor is transiently activated by heat treatment. Activated HSF1 binds to so-called heat shock element (HSE) sequences present in *hsp* genes. Mutants of human HSF1 were identified that transactivate *hsp* genes in the absence of an activating treatment (Zuo et al., 1995; Xia et al., 1999). One such mutant, HSF1(+), was made use of in the first regulatory circuit (Fig.1A). Note that in the experiments shown herein the gene of interest was substituted with a firefly luciferase gene. Because of the relative lability of the products of firefly luciferase genes, luciferase activity could be used as a reasonably sensitive indicator of changes in luciferase gene transcription. In cells containing the first circuit, there

should be essentially no transcription from *hsp70* gene promoters prior to an activating treatment, and the circuit should therefore be in the off state. Upon heat treatment, endogenous HSF1 (eHSF1) will be activated and begin transactivation of the HSF1(+) and luciferase genes. Subsequent to the heat treatment, unless it had been severe (lethal), the activity of eHSF1 will decline. Continued expression from both luciferase and HSF1(+) genes will become increasingly dependent on the activity of HSF1(+). An additional desirable feature of the circuit was that it could potentially adopt different levels of activity depending on the intensity of the activating treatment. Pure autoactivation or feed forward loops are inherently unstable and have at most two stable states of activity, i.e., inactive and fully active. Comparison of amounts of HSF1(+) and wildtype HSF1 synthesized over a two-day period by cells transfected with the respective expression constructs (not shown) suggested that HSF1(+) is a relatively labile protein. Hence, in the present circuit, the HSF1(+) autoactivation loop could be expected to be counterbalanced by substantial degradation and, possibly, inactivation of HSF1(+). Conceivably, therefore, the circuit could be capable of assuming multiple stable states of activity. The circuit was tested in cultures of a human HeLa or a mouse NIH 3T3 (not shown) cell line co-transfected with constructs hsp70-Luc and hsp70-HSF1(+) or, as a control, vector pcDNA3.1. The circuit was found to have the predicted properties (Fig.1B): in untreated cells, luciferase activity remained very low for the entire duration (5 days) of the experiment. Luciferase activity increased proportionally with the length of the activating heat treatment and was much higher in cells containing the circuit than in control cells. Most importantly, the activated circuit remained active during the remainder of the observation period. In the experiment shown, luciferase activity only decreased about two fold over the four-day period following activation. In other experiments (not shown), it either remained unchanged or increased somewhat (by two fold or less). Except for the residual activity seen in cells subjected to the longest activating treatment (60 min), luciferase activity in control cells returned to background level within

the observation period. Similarly sustained activity was also observed for the other circuits discussed below.

The intensity of a heat treatment is a function of both temperature and length of exposure to an elevated temperature (examined in the previous experiment). In the experiment in Fig.2, circuit activity was tested as a function of temperature of the activating treatment. Sustained levels of reporter expression directed by the circuit were found to increase with temperature between 42 and 45^{o}C. Hence, circuit activity was proportional to the intensity of the activating heat treatment.

That the circuit remained active for a period of more than four days suggested that its activity was maintained by HSF1(+), which factor was being continuously synthesized and, therefore, remained present in an effective concentration (Fig.1A). This interpretation of results depended on two assumptions, the first being that luciferase mRNA and protein turned over rapidly. To test this assumption, the effects on luciferase activity of 6-h exposures of cells containing luciferase genes under the control of an activated circuit to actinomycin D and cycloheximide were examined (Fig.3A). From the results it was calculated that luciferase activity declined with a half life of about 9 h in actinomycin D-treated cells and of about 7 h in cycloheximide-treated cells. Thus, reporter activities measured one to four days after activating treatment largely reflected recent reporter gene expression. The second assumption was that circuit activity could not have been maintained for a four-day period only by the HSF1(+) synthesized in response to the activating treatment. The experiment that confirmed the validity of this assumption took advantage of an earlier observation that plasmid-borne viral promoters tend to be inactivated relatively rapidly. The activity of a circuit that contained an HSF1(+) gene driven by a CMV promoter instead of the *hsp70B* gene promoter was followed for several days following activation (Fig.3B).

Circuit activity declined rapidly; three days after transfection, the remaining activity was about 2% of that on day one.

Experiments were also conducted with a second circuit that was identical to that outlined in Fig.1A except that the hsp70-Luc construct was substituted by a construct containing a human growth hormone (hGH) cDNA gene functionally linked to the *hsp70B* promoter (p17hGHdhfr; Dreano et al., 1986). Such a circuit was described in Example 2 of the referenced application. Cells containing this circuit produced, subsequent to heat activation, elevated levels of hGH during the entire experimental period of 5 days. No hGH was detected when the circuit was not heat-activated. Cells transfected with hsp70-hGH alone only expressed minimal amounts of hGH subsequent to the day of heat activation (not shown).

Type 2 Circuit

As is explained on pp.19-21 and in Fig.2 of the referenced application, type 2 circuits contain a mutated HSF in which the HSE DNA-binding domain is replaced by a different DNA-binding domain such as that of bacterial repressor LexA. Expression of the chimeric transcription factor is activatable by stress and by the chimeric factor itself. The gene of interest is controlled by a promoter that is responsive to the chimeric factor. Like a type 1 circuit, a type 2 circuit provides for sustained expression of the gene of interest in response to a single, transient stress. Unlike a type 1 circuit, however, a type 2 circuit does not result in concomitant sustained overexpression of endogenous Hsps.

The type 2 circuit outlined in Fig.4A was constructed. Note that this third circuit of this declaration is essentially the same circuit as that described on p.19, line 13 to p.20, line 2 of the referenced application. The only difference is that the circuit contains three instead of two components, corresponding to the embodiment of type 2 circuits described on p.21, lines 3-11, of the

application. Chimeric transactivator LexA-HSF1(+) was derived from HSF1(+) by replacement of the HSF1 DNA-binding domain with that of bacterial repressor LexA (Zuo et al., 1994; 1995). In cells containing the circuit, heat treatment was expected to activate the hsp70-LexA-HSF1(+) gene. Newly synthesized LexA-HSF1(+) should transactivate the lexA-Luc reporter gene as well as the lexA-LexA-HSF1(+) gene. Expression from the lexA-LexA-HSF1(+) gene should serve to maintain a LexA-HSF1(+) concentration sufficient for the continued transactivation of the lexA-Luc gene. The operation of the circuit was tested using HeLa cell cultures containing either the complete circuit or an incomplete circuit lacking the lexA-LexA-HSF1(+) construct. The unactivated circuit was only weakly active (Fig.4B). Heat treatment at 42^{o}C for 1 h increased its activity by at least an order of magnitude. Comparison of the activities of a complete (Fig.4B, left) and an incomplete (Fig.4B, right) circuit, measured three days after activating treatment, revealed the important contribution of replacement synthesis of transactivator. Thus, the circuit operated as intended to provide for heat-activated, sustained expression of a gene of interest following a single, transient stress, presumably without causing any sustained changes in the activities of endogenous *hsp* genes. It is noted that this three-component circuit and those discussed below may be readily converted to two-component systems by using bifunctional promoters (Lawson et al., 1985; Morganelli et al., 1985) to control transactivator expression.

Type 3 circuit

Type 3 circuits are described on pp.21-23 of the referenced application. The components of type 3 circuits are described on p.21, lines 16-28 (and in Fig.3), and an example is discussed on p.22, lines 4-30 and in Fig.4. The type 3 circuits resemble the type 2 circuits, except that they contain a transcription factor that is not derived from HSF. Note that embodiments of type 3 circuits were discussed in the referenced application on p.21, line 29 to p.22, line 3

and in example form on p.22, lines 4-30, and in Fig.4 that are regulated by stress and a second stimulus and, thus, are reversibly activatable.

In the experiments shown below two circuits comprising a ligand-regulated transcription factor were tested that were to mediate, in the presence of ligand, sustained expression of a gene of interest following activation by a single, transient heat treatment and to be inactivated following ligand withdrawal. The first circuit of this kind (the fourth circuit of this declaration) included chimeric transcription factor GLVP (Fig.5A) (Wang et al., 1994, 1997a & b). GLVP contains a GAL4 DNA-binding domain, VP16 transactivation domains and a progesterone receptor-derived ligand-binding domain. GLVP can be activated by anti-progesterone mifepristone (RU486). Upon heat treatment of cells containing this circuit, GLVP should be first expressed from construct hsp70-GLVP. In the presence of mifepristone (RU486), newly synthesized GLVP should transactivate both the 17x4-GLVP and 17x4-Luc genes. GLVP synthesis and, therefore, circuit activity should be maintained by autoactivated expression from the 17x4-GLVP gene. Results from a typical experiment are shown in Fig.5B. Sustained, elevated circuit activity was only detected in cells that had been subjected to an activating heat treatment and were cultured in the presence of mifepristone. Activity was proportional to the intensity of the activating treatment. The experiment in Fig.5C provided evidence for the reversibility of the circuit: a four fold reduction of activity was observed three days after mifepristone withdrawal.

The second circuit of this type (the fifth circuit of this declaration) was based on heterodimeric ponasterone A-regulated receptor VgEcR/RXR (Fig.6A) (No et al., 1996). Note that this circuit is essentially identical to that described in the referenced application (except that the RXR gene was expressed constitutively). See p.22 and Fig.4. This circuit displayed similar properties as the GLVP circuit (Fig.6B): high-level, sustained activity of the gene of interest (*luc*) was only observed subsequent to an initiating heat treatment and in the

presence of hormone ligand. Activity increased proportionally with the intensity of the activating treatment. Comparison of the activities of the complete circuit and a circuit lacking the E/GRE-VgEcR component confirmed that replacement synthesis of VgEcR contributed substantially to circuit activity. Inactivation of the gene of interest could be initiated by ligand withdrawal (Fig.6C).

In conclusion, five regulatory circuits of the invention were prepared and analyzed. The circuits were of all three types claimed in the referenced application and utilized four different transcription factors. To make each circuit, elements known in the art were combined according to the instructions provided in the referenced application using nothing more than published information on the transcription factors and target promoters and employing routine subcloning technology. Each circuit was found to display the properties described in the referenced application for the circuits of the invention: each provided for sustained activation of a gene of interest subsequent to a transient activating stress.

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6. I declare further that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified patent application or any patent issuing thereon.

APRIL 11, 01

Date

Richard Voellmy

Richard Voellmy, Ph.D.

CAPTIONS TO FIGURES

Fig.1. The first circuit. (A) The circuit consisted of construct hsp70-HSF1(+) containing a gene for transcription factor HSF1(+) functionally linked to the human *hsp70(B)* gene promoter and of construct hsp70-Luc containing the gene of interest (here luciferase) controlled by the same *hsp70B* promoter. eHSF1 refers to endogenous HSF1. The arrow leading away from the transcription factors is meant to indicate their degradation and/or inactivation. (B) Performance of the circuit. Parallel cultures in 60mm dishes were transfected by incubation for 5 h with 3 ml of Optimem containing 0.5 ug hsp70-Luc, 0.5 ug hsp70-HSF1(+) or pcDNA3.1 and 2 ug carrier (Bluescript) DNA, and 15 ul Lipofectamine^{TM} reagent. Subsequent to replacement of the transfection solution with fresh medium (DMEM with 10% fetal bovine serum), cultures were incubated at 37^{o}C for 16 h, subjected to heat treatment at 43.5^{o}C for 15, 30 or 60 min and incubated further at 37^{o}C for the indicated periods of time. Cultures were then harvested, and luciferase activities determined as described in Materials and Methods. Luciferase activities are expressed relative to the activity in cells cotransfected with hsp70-luc and pcDNA3.1 and kept at 37^{o}C ("fold induction").

Fig.2. Activity of the first circuit as a function of temperature of the activating treatment. Parallel cultures were co-transfected with constructs hsp70-Luc and hsp70-HSF1(+), pcDNA3.1 or (for comparison purposes) CMV-HSF1(+) as in Fig.1. Luciferase activity was measured 2 days after activating treatment. See Fig.1 for other experimental details.

Fig.3. (A) Turnover of luciferase mRNA and protein. Parallel cultures co-transfected with constructs hsp70-Luc, hsp70-HSF1(+) and carrier DNA as in Fig.1 were heat-treated at 42^{o}C for 60 min. One day after heat treatment, cultures were exposed for 6 h to either actinomycin (10 ug/ml), cycloheximide (50 ug/ml) or vehicle (0.1% ethanol). Luciferase activities were determined

immediately afterwards. Luciferase activities are expressed relative to the activity in cells cotransfected with hsp70-luc and pcDNA3.1 and kept at 37°C ("fold induction"). (B) Inactivation of a circuit that was identical to the first circuit except that HSF1(+) was expressed from a gene controlled by a CMV intermediate-early promoter instead of by an *hsp70B* gene promoter. Parallel cultures cotransfected with constructs hsp70-Luc, CMV-HSF1(+) and carrier DNA as in Fig.1 were incubated for the times indicated and then assayed for luciferase activity. Luciferase activities are expressed relative to the activity measured at 24 h (100%).

Fig.4. The third circuit. (A) The circuit consisted of (a) construct hsp70-LexA-HSF1(+) containing a gene for chimeric transcription factor LexA-HSF1(+) functionally linked to the human *hsp70B* promoter, (b) construct lexA-LexA-HSF1(+) containing the same transcription factor gene under the control of a promoter (lexA) responsive to the transcription factor, and (c) construct lexA-Luc containing the gene of interest (here luciferase) controlled by promoter lexA. eHSF1 refers to endogenous HSF1. The arrow leading away from transcription factors is meant to indicate their degradation and/or inactivation. (B) Performance of the circuit. Cultures in 6-well plates were transfected by incubation for 5 h with 1 ml Optimem containing 0.23 ug lexA-Luc, 46 ng hsp70-LexA-HSF1(+), 9.2 ng lexA-LexA-HSF1(+) or pcDNA3.1 and 1.09 ug carrier DNA, and 5 ul Lipofectamine™ reagent. Subsequent to replacement of transfection solution with fresh medium, cultures were incubated for 16 h at 37°C, subjected to heat treatment for 1 h at 42°C and incubated further for 3 days at 37°C. Cultures were then harvested, and luciferase activities determined as described in Materials and Methods. Luciferase activities are expressed relative to the activity in cells cotransfected with lexA-Luc, hsp70-LexA-HSF1(+) and pcDNA3.1 and kept at 37°C ("fold induction").

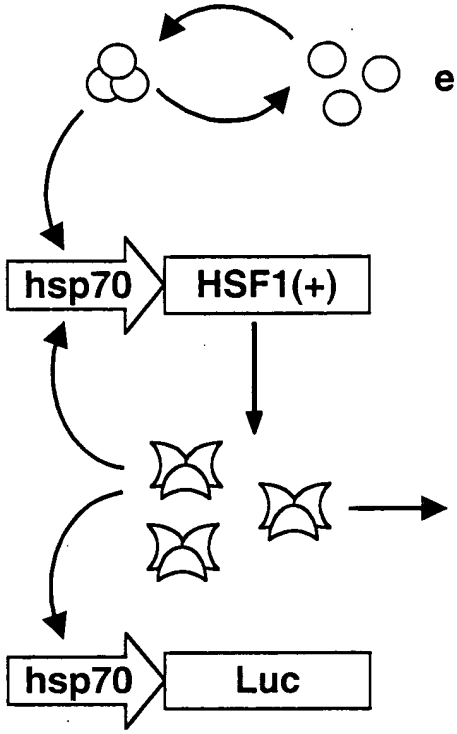
Fig.5. The fourth circuit. (A) The circuit consisted of (a) construct hsp70-GLVP containing a gene for transcription factor GLVP functionally linked to the human *hsp70B* promoter, (b) construct 17x4-GLVP containing the same transcription factor gene under the control of a promoter (17x4) responsive to the transcription factor, and (c) construct 17x4-Luc containing the gene of interest (here luciferase) controlled by promoter 17x4. eHSF1 refers to endogenous HSF1. The arrow leading away from transcription factor is meant to indicate its degradation and/or inactivation. (B) Performance of the circuit. Cultures in 12-well plates were transfected by incubation for 5 h with 0.5 ml Optimem containing 0.4 ug 17x4-Luc, 50 ng hsp70-GLVP, 50 ng 17x4-GLVP or pcDNA3.1 and 2 ul Lipofectamine^{TM} reagent. Subsequent to replacement of transfection solution with fresh medium, cultures were incubated for 16 h at 37^{o}C and then treated as indicated below the graph. Cultures were exposed to 10 nM mifepristone (Mif) or vehicle (0.1% ethanol). Heat treatment (HS) for 1 or 2 h at 43^{o}C was initiated 1 h after addition of Mif. Following 3 days of further incubation at 37^{o}C, cultures were harvested, and luciferase activities determined as described in Materials and Methods. (C) Deactivation of the circuit. Cultures transfected and incubated as above were exposed to the treatments indicated below the graph. Mifepristone was used at 1 nM, and heat shock was for 2 h at 43^{o}C. Cultures were postincubated for 3 days, except that one set of cultures was washed 8 h after heat treatment and incubated further in the absence of Mif (*). Luciferase activities are expressed relative to the activity in cells cotransfected with 17x4-Luc, hsp70-GLVP and 17x4-GLVP and kept at 37^{o}C in the presence of mifepristone ("fold induction").

Fig.6. The fifth circuit. (A) The circuit consisted of (a) construct hsp70-VgEcR-RXR containing a gene for transcription factor subunit VgEcR functionally linked to the human *hsp70B* promoter as well as a constitutively active RXR gene, (b) construct E/GRE-VgEcR containing the same VgEcR gene under the control of a promoter (E/GRE) responsive to transcription

factor VgEcR/RXR, and (c) construct E/GRE-Luc containing the gene of interest (here luciferase) controlled by promoter E/GRE. eHSF1 refers to endogenous HSF1. The arrow leading away from transcription factor is meant to indicate its degradation and/or inactivation. (B) Performance of the circuit. Cultures in 12-well plates were transfected by incubation for 5 h with 0.5 ml Optimem containing 0.4 ug E/GRE-Luc, 0.1 ug hsp70-VgEcR/RXR, 0.1 ug E/GRE-VgEcR or pcDNA3.1 and 2.4 ul Lipofectamine^{TM} reagent. Subsequent to replacement of transfection solution with fresh medium, cultures were incubated for 16 h at 37^{o}C and then treated as indicated below the graph. Cultures were exposed to 5 uM ponasterone A (P) or vehicle (0.05% ethanol). Heat treatment (HS) for 1 h at 42^{o}C or 43^{o}C, or for 30 min at 45^{o}C, was initiated 1 h after addition of hormone analog. Following 3 days of further incubation at 37^{o}C, cultures were harvested, and luciferase activities determined as described in Materials and Methods. (C) Deactivation of the circuit. Cultures transfected and incubated as above were exposed to the treatments indicated below the graph. Ponasterone A was used at 5 uM, and heat shock was for 30 min at 45^{o}C. Cultures were postincubated for 3 days, except that one set of cultures was washed 8 h after heat treatment and incubated further in the absence of hormone analog (*). Luciferase activities are expressed relative to the activity in cells cotransfected with E/GRE-Luc, hsp70-VgEcR-RXR and E/GRE-VgEcR and kept at 37^{o}C in the presence of ponasterone A ("fold induction").

Fig. 1

A


Transient Heat
eHSF1
hsp70
HSF1(+)
hsp70
Luc

B

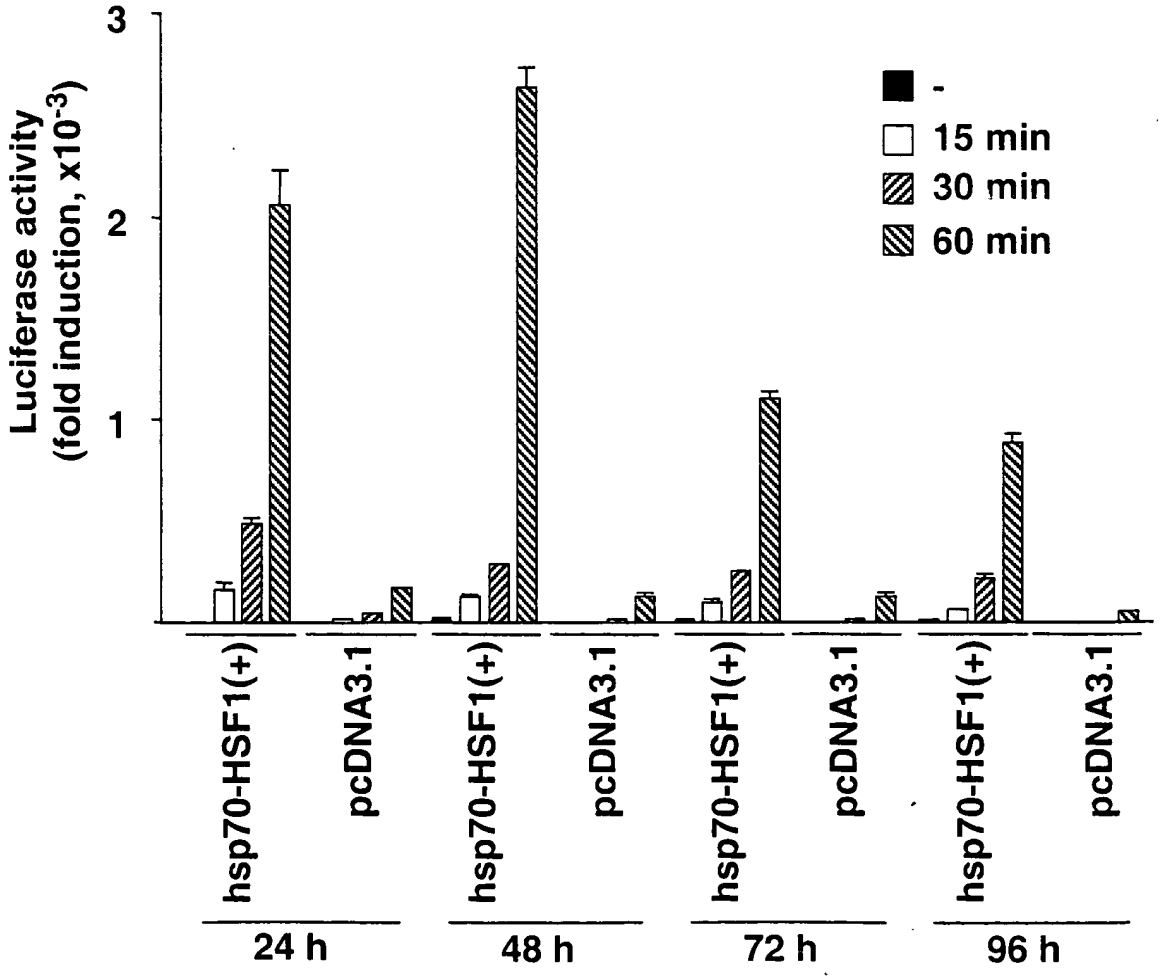

Luciferase activity
(fold induction, x10-3)
3
2
1
-
15 min
30 min
60 min
hsp70-HSF1(+)
pcDNA3.1
hsp70-HSF1(+)
pcDNA3.1
hsp70-HSF1(+)
pcDNA3.1
hsp70-HSF1(+)
pcDNA3.1
24 h
48 h
72 h
96 h

Fig. 2

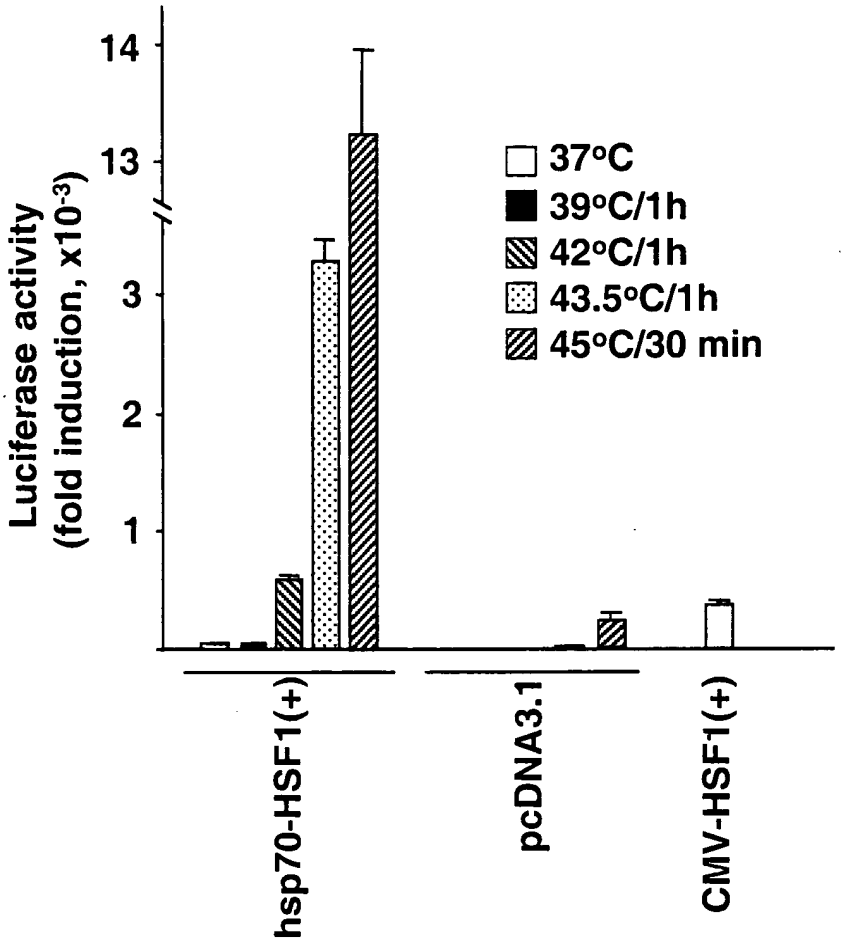

Luciferase activity
(fold induction, x10-3)
14
13
3
2
1
37°C
39°C/1h
42°C/1h
43.5°C/1h
45°C/30 min
hsp70-HSF1(+)
pcDNA3.1
CMV-HSF1(+)

Fig. 3

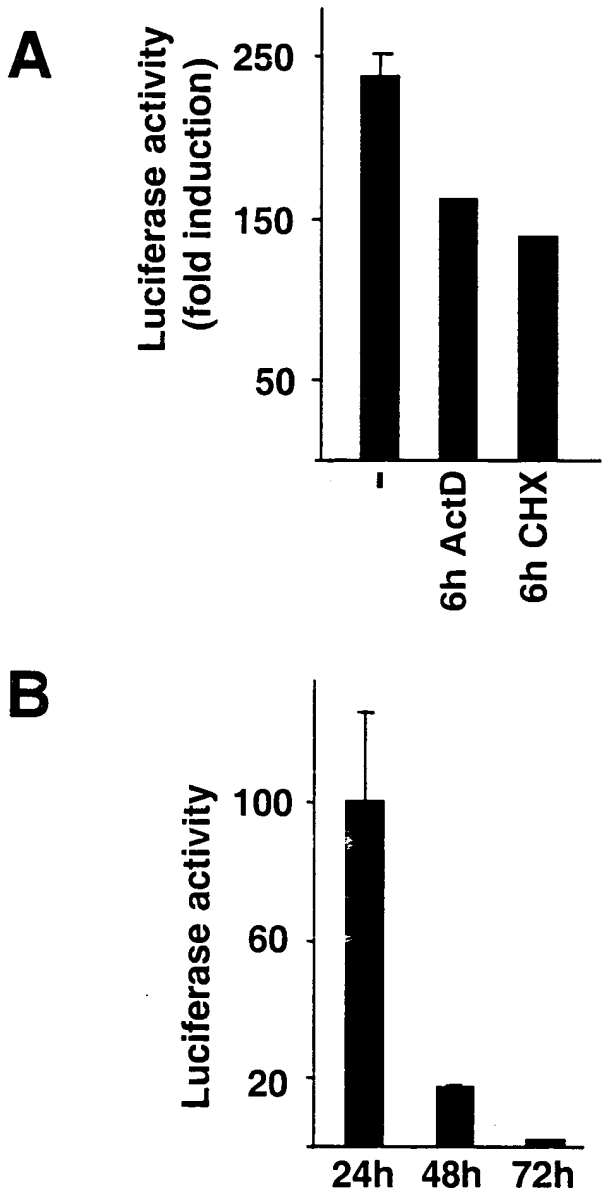
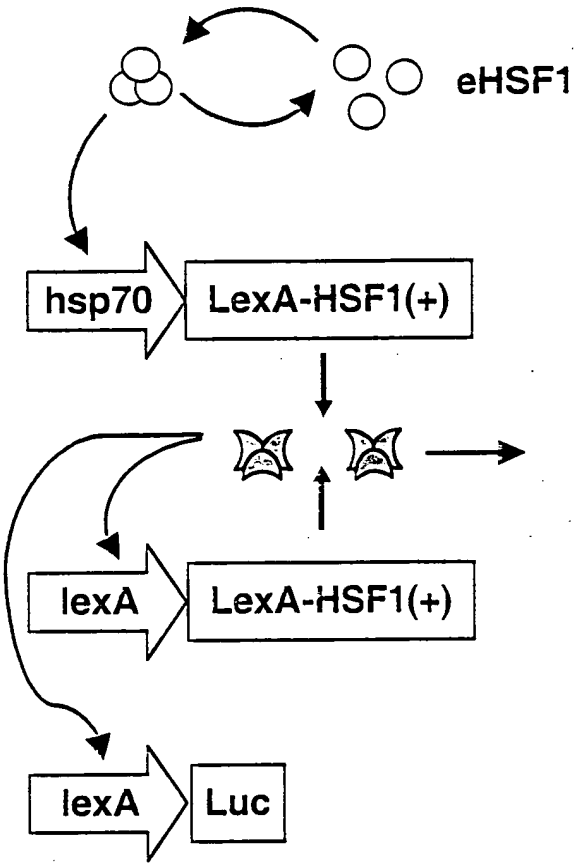

A
Luciferase activity
(fold induction)
250
150
50
-
6h ActD
6h CHX
B
Luciferase activity
100
60
20
24h
48h
72h

Fig. 4

A


Transient Heat
eHSF1
hsp70
LexA-HSF1(+)
lexA
LexA-HSF1(+)
lexA
Luc

B

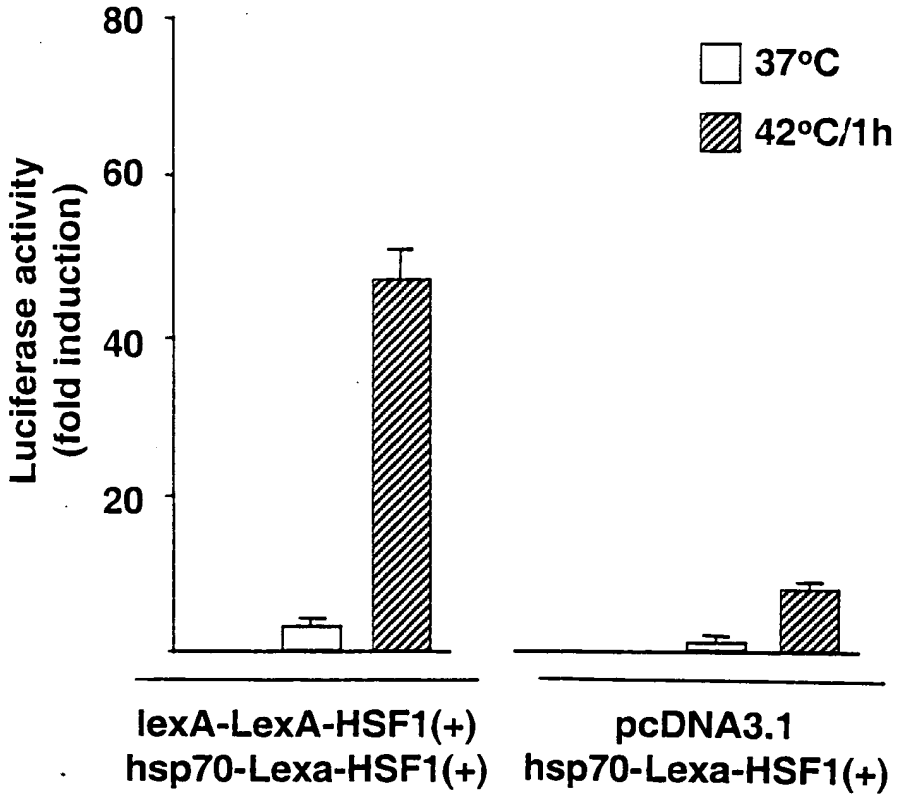

Luciferase activity
(fold induction)
80
60
40
20
37°C
42°C/1h
lexA-LexA-HSF1(+)
hsp70-Lexa-HSF1(+)
pcDNA3.1
hsp70-Lexa-HSF1(+)

Fig. 5

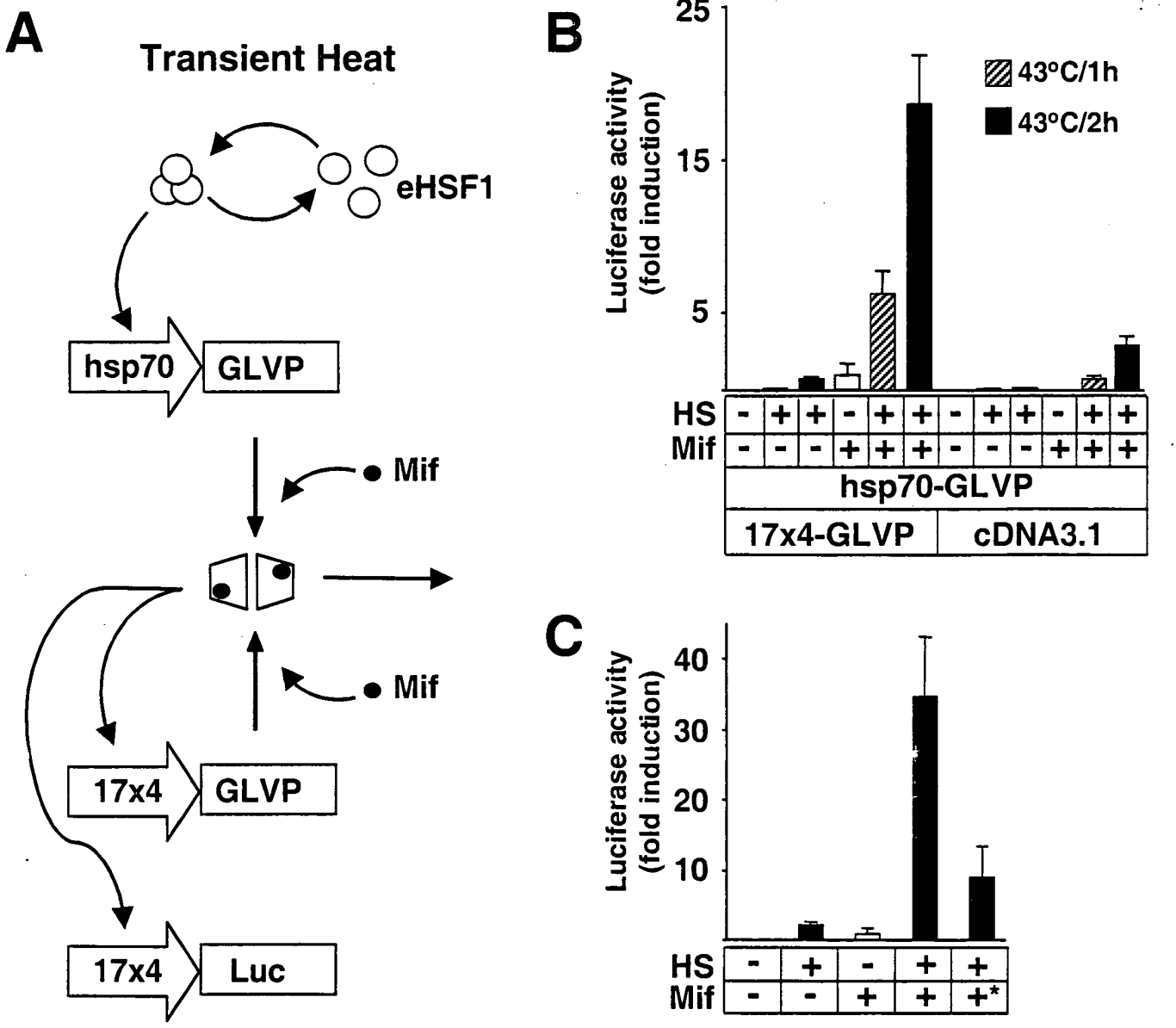
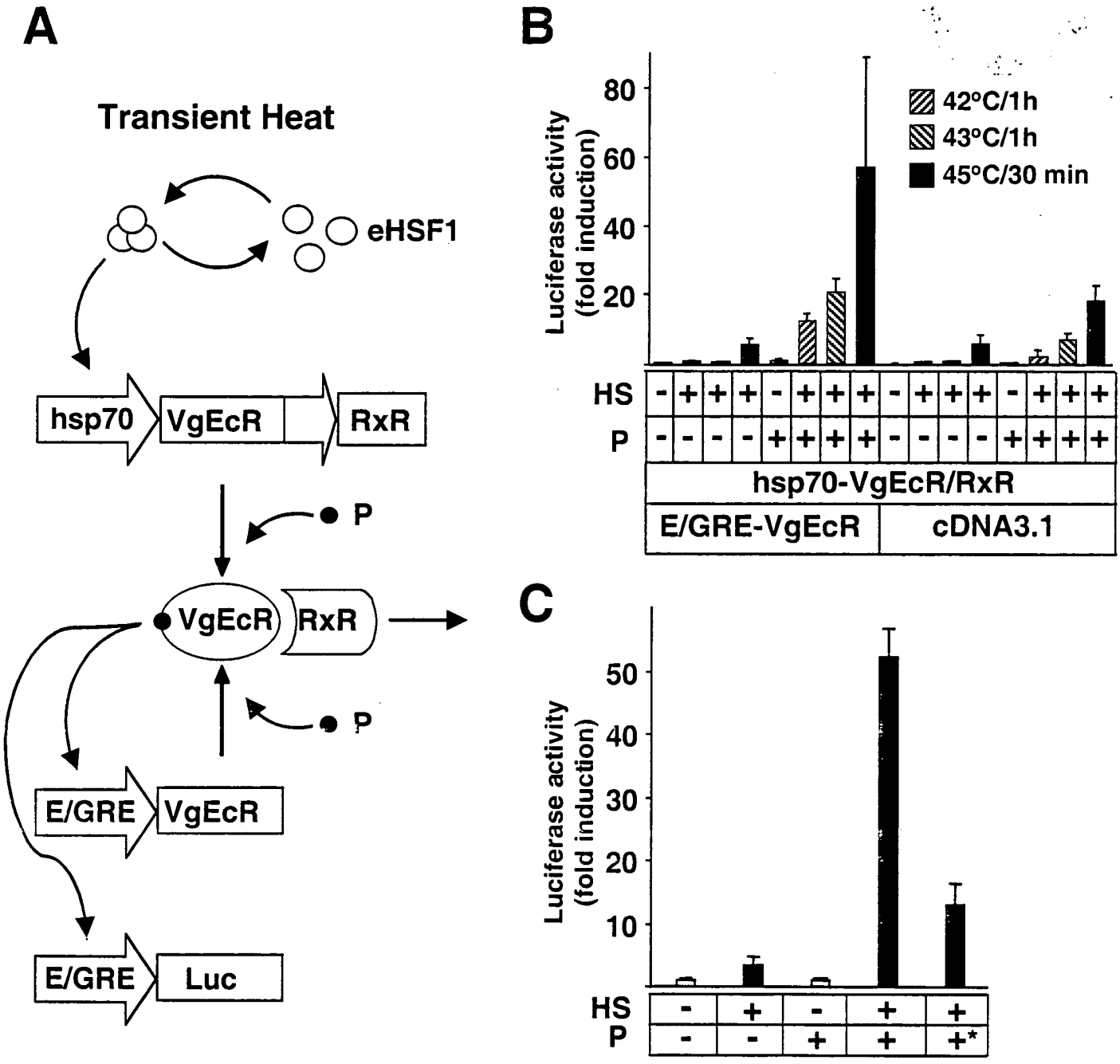

A
Transient Heat
eHSF1
hsp70
GLVP
Mif
Mif
17x4
GLVP
17x4
Luc
B
Luciferase activity (fold induction)
25
15
5
43°C/1h
43°C/2h
HS
Mif
hsp70-GLVP
17x4-GLVP
cDNA3.1
C
Luciferase activity (fold induction)
40
30
20
10
HS
Mif

Fig. 6


A
Transient Heat
eHSF1
hsp70
VgEcR
RxR
P
VgEcR
RxR
P
E/GRE
VgEcR
E/GRE
Luc
B
Luciferase activity (fold induction)
80
60
40
20
42°C/1h
43°C/1h
45°C/30 min
HS
P
hsp70-VgEcR/RxR
E/GRE-VgEcR
cDNA3.1
C
Luciferase activity (fold induction)
50
40
30
20
10
HS
P
+*